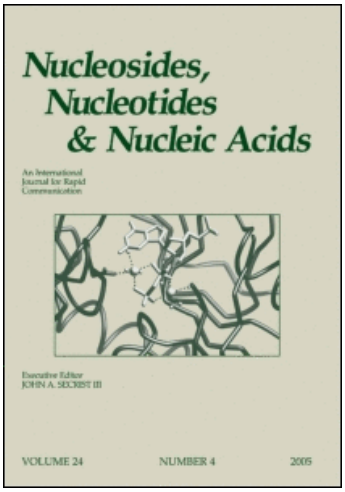

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A. K. Fotoohi[a]; M. Lindqvist[b]; C. Peterson[b]; F. Albertioni[a]
[a] Department of Oncology and Pathology, Cancer Center Karolinska (CCK), Karolinska Hospital, Stockholm, Sweden [b] Department of Medicine and Care, Faculty of Health Science, Linköping University, Linköping, Sweden

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IMPAIRED TRANSPORT AS A MECHANISM OF RESISTANCE TO THIOPURINES IN HUMAN T-LYMPHOBLASTIC LEUKEMIA CELLS

A. K. Fotoohi □ *Department of Oncology and Pathology, Cancer Center Karolinska (CCK), Karolinska Hospital, Stockholm, Sweden*

M. Lindqvist and C. Peterson □ *Department of Medicine and Care, Faculty of Health Science, Linköping University, Linköping, Sweden*

F. Albertioni □ *Department of Oncology and Pathology, Cancer Center Karolinska (CCK), Karolinska Hospital, Stockholm, Sweden*

□ *In order to better understand the mechanisms of resistance to thiopurines, we studied two sublines of the MOLT4 T-lymphoblastic leukemia cell line, resistant to 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG). We found that the underlying mechanism of resistance in both resistant cell lines was a markedly reduction in initial transport of 6-MP (3- and 5-fold, respectively, in 6-MP- and 6-TG-resistant cells). No significant alteration of activities of hypoxanthine-guanine phosphoribosyl transferase, thiopurine methyltransferase or inosine monophosphate dehydrogenase, the key enzymes involved in the metabolism of thiopurines was detected. We conclude that defected initial transport of thiopurines by cells may very well explain their resistance to these drugs.*

Keywords 6-Mercaptopurine; 6-Thioguanine; Resistance; Leukemia; Transport

INTRODUCTION

6-Mercaptopurine (6-MP) is one of the first developed antineoplastic agents and is a routine component of modern protocols for maintenance therapy of children with acute lymphoblastic leukemia (ALL). 6-Thioguanine (6-TG) is administered to induce remission, as well as for maintenance therapy of acute myelogenous leukemia (AML). As the cytotoxicity of these compounds is exerted by active metabolites formed

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Address correspondence to Alan Kambiz Fotoohi, Department of Oncology and Pathology, Cancer Center Karolinska (CCK), Karolinska Hospital, Stockholm, Sweden. E-mail: Alan.fotoohi@ki.se

intracellularly, they can be regarded as inactive prodrugs.[1] The active intracellular metabolites of thiopurines either inhibit purine de novo synthesis and/or are incorporated as nucleotides into RNA/DNA.[2] Intracellular metabolism of 6-MP is mediated by phosphoribosylation of 6-MP by the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) into 6-thioinosine 5′ monophosphate (TIMP) and is subsequently converted to thioguanosine monophosphate by a two-step process involving inosine monophosphate dehydrogenase (IMPDH) and guanosine monophosphate synthetase.[2] Methylation of TIMP by the polymorphic enzyme thiopurine methyltransferase (TPMT) results in methylthioinosine monophosphate (meTIMP) that is a strong inhibitor of purine de novo synthesis and induces cytotoxicity by depletion of ribonucleotides.[3] 6-TG is converted directly into 6-thioguanosine 5′-monophosphate through the enzyme HGPRT, and then to 6-thioguanosine 5′-di/tri phosphate which contributes to cytotoxicity when incorporated to DNA or RNA.[2] A recent study shows that TGNs as metabolites of azathioprine induce apoptosis of T cells from patients with Crohn disease by targeting the CD28-dependent Rac1 activation.[4]

The most extensively characterized mechanism of resistance to 6-MP and 6-TG is a decrement or lack of HGPRT activity. [5] Alterations in TPMT activity have been shown to influence the sensitivity of cells to the cytotoxic effects of both 6-MP and 6-TG, but in opposite directions.[6]

In the present study we have investigated the mechanisms of resistance of the human acute T-lymphoblastic leukemia MOLT4 cell line to 6-MP and 6-TG. We demonstrate here that rather than alterations in activities of metabolic enzymes, a reduced influx of 6-MP into MOLT4 cells can be the main mechanism of resistance to thiopurines.

MATERIALS AND METHODS

Cell Culture Conditions and Establishment of Resistant Cell Lines

The MOLT4 an acute T-lymphoblastic leukemia cell line, was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were subcultured twice weekly in RPMI-1640 medium supplemented with fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) under an atmosphere of humidified air containing 5% CO_2 at 37°C. To select the 6-MP- and 6-TG- resistant sub-clones from the parental MOLT4 cell line, the concentrations of these compounds in the culture medium were increased stepwise until the final concentration of 5 μM during a period of 4 months. The resistant cells were cultured in drug-free medium for at least 3 passages before using them for the experiments. The cells were counted utilizing Coulter Multisizer (Coulter Electronics, Luton, UK).

The cytotoxicity assay was performed by exposing log phase MOLT4 cells to different concentrations of drugs for 72 hours using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide) (Sigma, St. Louis, MO, USA) assay.

Transport Assay

Uptake and intracellular accumulation of 6-MP were measured by incubating logarithmically growing cells in the presence of [^{14}C] 6-MP at the time points indicated. Briefly, the cells in their logarithmic phase of growth were collected by centrifugation, washed and resuspended in Hepes-buffered RPMI-1640 medium (2×10^6 cells/ml) and incubated with 10 μM [^{14}C] 6-MP at 37°C. The initial transport assays were performed within 1 minute.

Uptake of 6-MP was terminated at the various time-points by the addition of 10:1 volumes ice-cold PBS, following by 1,800 × g centrifugation for 5 minutes at 4°C and then washing 3 times with ice-cold PBS. The cell pellets were resuspended in 150 μl ethanol 70%, and then stirred and centrifuged for 10 minutes at 4000 × g. The radioactivity in resulting protein-free supernatant was determined by liquid scintillation counting.

HGPRT, IMPDH, and TPMT Enzyme Assays

HGPRT, IMPDH, and TPMT assays were performed according to Pieters et al.,[7] Proffitt et al.,[8] and Weinshilboum et al.,[9] respectively.

RESULTS AND DISCUSSION

In order to assess the mechanism underlying resistance to thiopurines, we selected two resistant cell lines by exposing the parental MOLT4 cells during 12–18 cycles (72 hours each) to stepwise increasing concentrations of 6-MP or 6-TG (from 0.05–5 μM). The resulting cell lines were similar to the parental cells with respect to growth rate and various cell cycle parameters (data not shown). Characterization of resistant cells employing a 72-hour exposure to 6-MP and 6-TG revealed at least 5-fold resistance to both 6-MP and 6-TG of 6-MP-resistant (MOLT4/MP) cells (IC_{50}s = 23.5 ± 16 and 18.3 ± 4.6 μM, respectively. The respective IC_{50}s for wild type MOLT4 cells were 3.3 ± 0.9 and 2.1 ± 0.4 μM). The cell line selected for resistance to 6-TG (MOLT4/TG) was >14–fold more resistant to both 6-TG and 6-MP (IC_{50}s = 48 ± 13 and 63 ± 21 μM, respectively) (Figures 1A and 1B).

Currently, there is no evidence supporting a significant contribution of low HGPRT activity to clinical resistance to thiopurines. However, the absence of HGPRT activity is the most extensively characterized mechanism

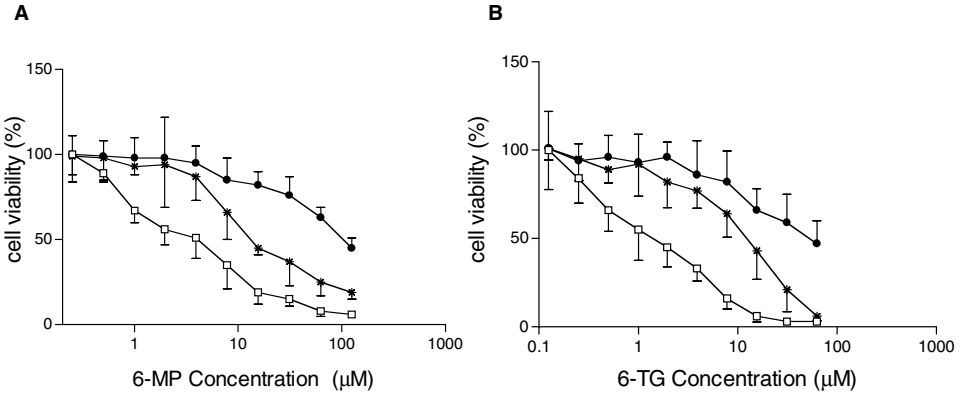

FIGURE 1 The growth inhibitory effects of 6-MP (A) and (B) on MOLT4/WT (□), MOLT4/6-MP (∗), and MOLT4/6-TG (■) cells. The cells were incubated with a range of concentrations of 6-MP and 6-TG for a period of 72 hours under a humidified atmosphere containing 5% CO_2 at 37°C. The results presented are the means ±S.D. of 3 independent experiments.

for in vitro resistance to 6-MP and 6-TG. However, very low level of HGPRT is sufficient to generate the cytotoxic nucleotides so that cells which become resistant via this mechanism express this enzyme at levels that are 100- to 1000-fold lower than in wild type cells.[7] The slight decrease in HGPRT activity observed in our thiopurine resistant cells can not explain the high levels of tolerance of these cells to 6-MP and 6-TG (Figure 2A). Neither of our resistant cell lines demonstrated any change in the levels of either TPMT activity in cell extracts nor did they exhibit any significant alteration in the activity of IMPDH. (Figure 2B and 2C).

Utilizing radiolabeled 6-MP, we measured the accumulation of 6-MP over a period of 60 minutes, as well as initial transport of 6-MP during seconds. In both assays the uptake was profoundly impaired in resistant cell lines compared to their wild type parents. The values of uptakes by the parental MOLT4 cells and 6-MP- and 6-TG-resistant sublines after 60 minutes were 18 ± 1, 4.3 ± 0.6 and 2.9 ± 0.5 nmol/10^7 cells, respectively (Figure 2D).

Ruling out alterations in intracellular metabolism by firstly determination of the same amounts of activities of key enzymes from one side, and secondly restricting the time points of transport assays to seconds, emphasizes that the >5-fold lower intracellular accumulation of 6-MP in our resistant cells can be solely because of a defect in uptake.

As analogues of hypoxanthine and guanine, it is probable that 6-MP and 6-TG act as substrates for nucleoside transporters. Determination of the probable alterations of transporter genes may be valuable to explain the mechanisms behind low transport of 6-MP in our resistant cells. Therefore, we are performing global gene expression analysis to detect any alteration of expression of the suspected genes.

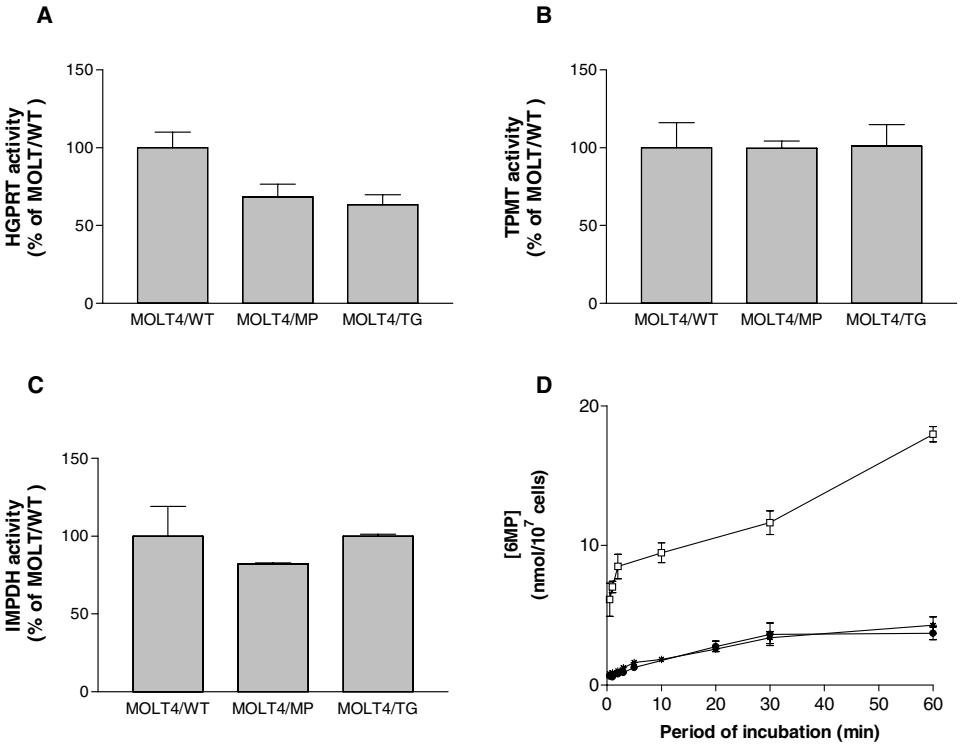

FIGURE 2 Activities of the enzymes HGPRT (A), TPMT (B), and IMPDH (C). Crude cell extracts used as sources of enzymes as explained in "Materials and Methods". 6-MP and 6-TG resistant cells demonstrated comparable levels of activities of all three enzymes as wild type cells. The results presented are the means ±S.D. of at least 3 independent experiments. D. Transport of [^{14}C]6-MP into MOLT4/WT (●), MOLT4/6-MP (∗), and MOLT4/6-TG (■) cells as a function of time. The concentration of [^{14}C]6-MP in the medium was 10 μM. The results presented in this figure are the means ±S.D. of 3 independent experiments.

In summary, in MOLT4 cells selected for resistance to 6-MP and 6-TG, defective cellular uptake could account for underling mechanism of resistance. No significant alteration was detected in activities of HGPRT, TPMT and IMPDH enzymes involved in metabolism pathway of thiopurines.

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